

AD _____

Award Number: DAMD17-03-1-0265

TITLE: Exploiting the Innate Antitumor Activity of Human
Gamma-Delta T-Cells for the Treatment of Prostate Cancer

PRINCIPAL INVESTIGATOR: Richard D. Lopez, M.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20041118 078

BEST AVAILABLE COPY

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Apr 2003 - 31 Mar 2004)	
4. TITLE AND SUBTITLE Exploiting the Innate Antitumor Activity of Human Gamma-Delta T-Cells for the Treatment of Prostate Cancer			5. FUNDING NUMBERS DAMD17-03-1-0265	
6. AUTHOR(S) Richard D. Lopez, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alabama at Birmingham Birmingham, AL 35294 E-Mail: Richard.Lopez@ccc.UAB.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: ALL DTIC reproductions will be in black and white				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We initially identified and characterized a CD2-mediated, interleukin (IL)-12-dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human $\gamma\delta$ -T cells. We have since exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable <i>apoptosis-resistant</i> $\gamma\delta$ -T cells. We have shown that apoptosis-resistant human $\gamma\delta$ -T cells retain significant innate, major histocompatibility complex (MHC)-unrestricted cytotoxicity against human prostate cancer cell lines. Purpose and scope: The aims of this project are, 1) to more precisely characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant human $\gamma\delta$ -T cells against human prostate cancer cells; 2) to define the general mechanisms involved in the <u>recognition</u> and <u>lysis</u> of sensitive prostate cancer cells by apoptosis-resistant $\gamma\delta$ -T cells; and 3) to determine the extent to which apoptosis-resistant $\gamma\delta$ -T cells can regulate the growth and metastasis of prostate cancer cells <i>in vivo</i> . Key findings to date: 1) $\gamma\delta$ -T cells derived from a variety of donors consistently displayed lytic activity against prostate cancer cell lines DU-145 and PC-3, but not LNCaP. 2) Monoclonal antibodies (mAb) against either the V γ 9 or V δ 2 TCR chains as well as mAb against intercellular adhesion molecules-1 (ICAM-1) or CD18 (β subunit of the β_2 integrins) blocked $\gamma\delta$ -T cell-mediated killing of prostate cancer cells; 3) $\gamma\delta$ -T cells lyse prostate cancer cell lines largely through the perforin/granzyme pathway. 4) Using the TRAMP transgenic mouse model of prostate cancer, we have shown that the absence of $\gamma\delta$ -T cells is indeed permissive for the development of tumors.				
14. SUBJECT TERMS Prostate cancer; immunotherapy; cellular therapy, gamma/delta T cell				15. NUMBER OF PAGES 20
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	6
Appendices.....	7

INTRODUCTION

We initially identified and characterized a CD2-mediated, interleukin (IL)-12-dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human $\gamma\delta$ -T cells. We have since exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable *apoptosis-resistant* $\gamma\delta$ -T cells – an undertaking until now, not possible. Importantly, we have shown that apoptosis-resistant human $\gamma\delta$ -T cells retain significant innate, major histocompatibility complex (MHC)-unrestricted cytotoxicity against a wide variety of human-derived tumor cell lines, including human prostate cancer cell lines. Our efforts related to this proposal have remained focused upon testing the hypothesis that $\gamma\delta$ -T cells – by virtue of their innate ability to recognize and kill epithelial-derived malignancies – play an important role in regulating the initial growth or spread of prostate cancer in vivo. The specific aims of this project are, 1) to more precisely characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant human $\gamma\delta$ -T cells against human prostate cancer cells; 2) to define the general mechanisms involved in the recognition and lysis of sensitive prostate cancer cells by apoptosis-resistant $\gamma\delta$ -T cells; and 3) to determine the extent to which apoptosis-resistant $\gamma\delta$ -T cells can regulate the growth and metastasis of prostate cancer cells *in vivo*.

BODY

In the initial period of this grant for which this report is generated (April 1, 2003 to March 1, 2004) our accomplishments are presented in relation to the following tasks as outlined in the approved Statement of Work.

Task 1. To characterize the extent and breadth of the antitumor cytotoxicity mediated by apoptosis-resistant human $\gamma\delta$ -T cells against human prostate cancer cells

We have re-prioritized the order in which we are approaching our tasks. All patient-related studies have been moved back 12 months, as such, no results are presented here.

Task 2. To define the general mechanisms involved in the recognition and lysis of sensitive prostate cancer cells by apoptosis-resistant $\gamma\delta$ -T cells.

Initial Objectives and Rationale. Here, we proposed to determine the extent to which various known molecular receptors are used by apoptosis-resistant $\gamma\delta$ -T cells to recognize sensitive prostate cancer cells. We also proposed to determine which molecular ligands present on sensitive prostate cancer cells (such as MICA or other MHC class-I-like molecules) are recognized by apoptosis-resistant $\gamma\delta$ -T cells. In addition, we proposed to define the extent to which known adhesion molecules are involved in the cellular interactions between apoptosis-resistant $\gamma\delta$ -T cells and sensitive prostate cancer cells. Finally, we proposed to determine the extent to which granule exocytosis or Fas/FasL-mediated mechanisms are utilized by apoptosis-resistant $\gamma\delta$ -T cells in the killing of sensitive prostate cancer cells.

Findings. The key findings related to this task are presented in the attached draft of the manuscript entitled, "*Ex vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells mediate innate antitumor activity against human prostate cancer cells in vitro*". This manuscript is to be submitted shortly. In summary, (taken from the manuscript) we have found:

- $\gamma\delta$ -T cells derived from a variety of donors consistently displayed lytic activity against prostate cancer cell lines DU-145 and PC-3, but not LNCaP.
- Monoclonal antibodies (mAb) against either the V γ 9 or V δ 2 TCR chains as well as mAb against intercellular adhesion molecules-1 (ICAM-1) or CD18 (β subunit of the β_2 integrins) blocked $\gamma\delta$ -T cell-mediated killing of prostate cancer cells.
- $\gamma\delta$ -T cells lysed prostate cancer cell lines largely through the perforin/granzyme pathway.

From these findings we can conclude that ex-vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells are able to innately recognize and kill certain human prostate tumor cell lines in vitro. Recognition and killing of prostate cancer cells occurs in a $\gamma\delta$ -TCR—dependent manner and also appears to involve adhesion occurring through ICAM-1 and CD18. The cytolytic process involves primarily the perforin/granzyme mediated pathway of granule exocytosis.

Task 3. To determine the extent to which apoptosis-resistant $\gamma\delta$ -T cells can regulate the growth and metastasis of prostate cancer cells in vivo

Initial Objectives and Rationale. Our initial intent of these studies was to use a transgenic mouse model of prostate cancer (TRAMP) to define the role of $\gamma\delta$ -T cells in controlling the development and growth of prostate cancer. This was to be accomplished by classical genetic means where TRAMP mice will be bred with $\gamma\delta$ -T cell knockout mice (TCR $\delta^{-/-}$) — animals which we then predict will develop tumors more rapidly and extensively. TRAMP-derived prostate cancer cell lines will also be used to establish tumors in syngeneic TCR $\delta^{-/-}$ mice. We then propose to rescue these animals by the adoptive transfer of syngeneic apoptosis-resistant murine $\gamma\delta$ -T cells (which we can also now readily generate and isolate).

Findings, 1. Using the TRAMP transgenic mouse model of prostate cancer, we have shown that the absence of $\gamma\delta$ -T cells is indeed permissive for the development of tumors as demonstrated in **Figure 1** (unpublished data). TRAMP mice develop prostate cancer in a predictable manner. By back-crossing TRAMP mice with mice lacking $\gamma\delta$ -T cells (TCR $\delta^{-/-}$), we observe that TRAMP \times TCR $\delta^{-/-}$ mice (**Figure 1C**) develop more aggressive prostate cancers at a far younger age in comparison to control TRAMP animals (**Figure 1B**).

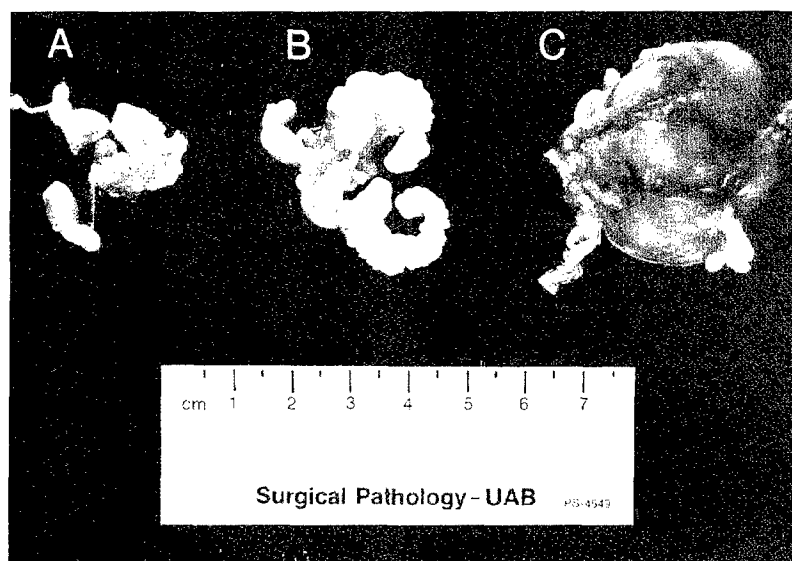


Figure 1. Absence of $\gamma\delta$ -T cells is permissive for the development of cancer. As described and referenced in the text, TRAMP mice are transgenic for a construct consisting of the minimal rat probasin promoter which drives expression of the SV40 early genes (T and t; *T_{ag}*) in a prostate tissue-specific manner. As a consequence, TRAMP mice spontaneously develop prostate adenocarcinomas in a predictable manner. In order to determine if the absence of $\gamma\delta$ -T cells is permissive for the development of adenocarcinoma, TRAMP mice were first backcrossed with commercially available TCR δ -chain knockout mice (TCR $\delta^{-/-}$ mice, Jackson Labs, Bar Harbor, ME) which fail to develop $\gamma\delta$ -T cells. **A)** Normal genitourinary (GU) tract of a male wild-type C57BL/6J mouse surgically removed at 7 months of age. **B)** GU tract of a male TRAMP mouse surgically removed at 7 months of age. Tumor infiltration of GU structures (including bladder, epididymis and prostate) were observed grossly and microscopically (not shown) in a manner consistent with published data. **C)** GU tract of a male TRAMP \times TCR $\delta^{-/-}$ animal removed at 5 months of age. Gross effacement of all GU structures was observed. Early sacrifice of TRAMP \times TCR $\delta^{-/-}$ animals was performed in accordance with animal welfare guidelines. Not shown, TRAMP \times TCR $\beta^{-/-}$ animals ($\alpha\beta$ -T cell-deficient) had GU tracts similar to age-matched TRAMP animals.

Findings, 2. We have also been able to transfer TRAMP cell lines into TCR $\delta^{-/-}$, TCR $\beta^{-/-}$ and control C57BL/6 mice. We are now treating these animals with mouse $\gamma\delta$ -T cells derived from wild-type C57BL/6 mice. We have no definitive results to report at this early time.

KEY RESEARCH ACCOMPLISHMENTS

- We can now conclude that ex-vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells are able to innately recognize and kill certain human prostate tumor cell lines in vitro. Recognition and killing of prostate cancer cells occurs in a $\gamma\delta$ -TCR—dependent manner and also appears to involve adhesion occurring through ICAM-1 and CD18. The cytolytic process involves primarily the perforin/granzyme mediated pathway of granule exocytosis.
- We have derived key animal data which support our view that the absence of $\gamma\delta$ -T cells is indeed permissive for the development of prostate cancer using the TRAMP mouse model. These are key proof-of-principal studies that support our overall hypothesis.

REPORTABLE OUTCOMES

- none to date

CONCLUSIONS

- As noted above, the findings that we report here are quite preliminary, but support our overall hypothesis that "By virtue of their ability to innately recognize and kill epithelial-derived malignancies, human $\gamma\delta$ -T cells play an important role in regulating the initial growth or spread of prostate cancer in vivo".

REFERENCES

- none

APPENDIX

- draft of manuscript entitled, "*Ex vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells mediate innate antitumor activity against human prostate cancer cells in vitro*", to be submitted shortly.

Ex vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells mediate innate antitumor activity against human prostate cancer cells in vitro

ABSTRACT

Purpose:

Unlike $\alpha\beta$ -T cells, $\gamma\delta$ -T cells recognize tumor cells in a major histocompatibility complex (MHC)-independent manner which requires no processing or presentation of tumor-specific antigens. Consequently, $\gamma\delta$ -T cells can recognize and lyse tumor cells almost immediately upon encounter – consistent with their role as a component of the innate immune system. Strategies designed to exploit this innate antitumor activity of human $\gamma\delta$ -T cells for the treatment of cancers, including prostate cancer, are appealing for this reason. We have previously identified a CD2-mediated, IL-12-dependent signaling pathway which inhibits activation-induced cell death (AICD) in mitogen-stimulated human $\gamma\delta$ -T cells which in turn permits the large-scale expansion of apoptosis-resistant $\gamma\delta$ -T cells, most of which express the V δ 2 T cell receptor (TCR) chain, in combination with the V γ 9 TCR chain. While it is well documented that $\gamma\delta$ -T cells which express the V δ 1 TCR chain display broad cytolytic activity against a variety of epithelial-derived human tumors, less is known regarding the antitumor activity of V γ 9V δ 2+ $\gamma\delta$ -T cells, particularly against epithelial-derived malignancies. Here we describe the innate antitumor activity of ex vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells against prostate cancer cells.

Materials and Methods:

Apoptosis resistant human $\gamma\delta$ -T cells were first expanded in vitro from cultured human peripheral blood mononuclear cells (PBMC) then enriched to high purity utilizing standard immunomagnetic separation technology. In vitro cytotoxicity of expanded apoptosis resistant $\gamma\delta$ -T cells was measured against human prostate cancer-derived cell lines and was determined utilizing standard four hour ^{51}Cr - release assay.

Results:

$\gamma\delta$ -T cells derived from a variety of donors consistently displayed lytic activity against prostate cancer cell lines DU-145 and PC-3, but not LNCaP. Monoclonal antibodies (mAb) against either the V γ 9 or V δ 2 TCR chains as well as mAb against intercellular adhesion molecules-1 (ICAM-1) or CD18 (β subunit of the β_2 integrins) blocked $\gamma\delta$ -T cell-mediated killing of prostate cancer cells. $\gamma\delta$ -T cells lysed prostate cancer cell lines largely through the perforin/granzyme pathway.

Conclusion:

Ex-vivo expanded human V γ 9V δ 2+ $\gamma\delta$ -T cells are able to innately recognize and kill certain human prostate tumor cell lines in vitro. Recognition and killing of prostate

cancer cells occurs in a $\gamma\delta$ -TCR-dependent manner and also appears to involve adhesion occurring through ICAM-1 and CD18. As apoptosis-resistant human V γ 9V δ 2+ $\gamma\delta$ -T cells can readily be expanded to large numbers in our laboratory, these findings must be considered in the context of developing new adoptive cellular therapy approaches for the treatment of prostate cancer.

Key Words:

$\gamma\delta$ -T cell; cytotoxicity; prostate cancer; innate immunity; adoptive immunotherapy.

INTRODUCTION

Adenocarcinoma of the prostate remains one of the leading causes of cancer-related deaths among American men. Although current standard therapies for early-stage prostate cancer – including surgery, radiotherapy or hormonal blockade – are usually very effective at achieving initial disease control, prostate cancer often recurs. Moreover, salvage chemotherapy for recurrent prostate cancer – or chemotherapy for prostate cancer presenting initially as wide-spread metastatic disease – is often associated with very poor responses. Clearly, new forms of therapy for recurrent or metastatic prostate cancer are needed.

The view that the immune system itself might be exploited for the treatment of prostate cancer is not new. Interestingly, to date the overwhelming majority of reports in this regard have focused primarily upon augmenting the adaptive immune response to prostate cancer-specific antigens. This includes a number of important studies designed to induce tumor-specific cytotoxic CD8+ $\alpha\beta$ -T lymphocytes (CTL) utilizing prostate cancer-specific peptide antigens, as well as other studies designed to develop tumor-specific immune responses employing dendritic cell-based vaccination strategies (1-15). However, these and similar approaches which rely primarily upon adaptive immunity suffer from several potential shortcomings.

First, these strategies presuppose that an antigen selected as a target for immune-based therapy is indeed, tumor-specific – that is, the antigen is expressed only in tumor cells, but not in normal tissues. This problem is illustrated when one considers studies designed to target prostate specific antigen (PSA) as a tumor-specific immunogen: While PSA is expressed by the vast majority of prostate cancer cells, it is also expressed by normal prostate tissues. Moreover, PSA might not be expressed at all by some poorly differentiated adenocarcinomas.

Secondly, it is well established that prostate cancer cells can either downregulate the expression of MHC class-I or class-II molecules, or suffer from defects in assembly and expression of MHC class-I (10, 16, 17). Thus, tumor cells expressing little or no MHC molecules might selectively escape recognition by MHC-restricted CD8+ CTL or CD4+ T helper cells. These latter findings emphasize the importance of developing immunotherapy strategies that do not require or even utilize classical MHC-restricted antigen processing and presentation.

Unlike $\alpha\beta$ -T cells, $\gamma\delta$ -T cells recognize tumor cells in an MHC-independent manner, requiring no processing or presentation of tumor-specific antigens. Instead, $\gamma\delta$ -T cells recognize a variety of MHC class-I-like antigens which are commonly displayed by cells having undergone malignant transformation, particularly cells of epithelial origin (18-20). Indeed, $\gamma\delta$ -T cells may provide an alternative or complementary means of recognizing and killing tumor cells which have escaped adaptive immune responses.

In peripheral blood, $\gamma\delta$ -T cells represent only a minor fraction of total T cells, usually only 1 to 5 percent (21). The vast majority of peripheral blood $\gamma\delta$ -T cells express the V γ 9 TCR chain usually in combination with the V δ 2 TCR chain (V γ 9V δ 2 $\gamma\delta$ -T cells, also termed V γ 2V δ 2 in an alternate nomenclature). It is thought that V γ 9V δ 2 $\gamma\delta$ -T cells provide some degree of immunosurveillance against intracellular pathogens and certain hematological malignancies (22-24). In contrast, $\gamma\delta$ -T cells found in epithelial tissues (such as the intestine, skin, tongue, esophagus, trachea, lungs and genital tract) usually express the V δ 1 TCR chain in combination with a variety of V γ chains (25). V δ 1 $\gamma\delta$ -T cells can be found in association with or even infiltrating some solid tumors and often display MHC-unrestricted lytic activity against a variety of cancer cells – particularly those of epithelial origin (25).

We have previously identified a CD2-mediated, IL-12-dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human $\gamma\delta$ -T cells derived from peripheral blood (26). This in turn allows for the large-scale expansion of apoptosis-resistant $\gamma\delta$ -T cells, most of which express the V γ 9V δ 2 TCR.

Although it is been reported that activated V γ 9V δ 2 $\gamma\delta$ -T cells are capable of in vitro killing of human lymphoma cell lines, little is known regarding the innate antitumor activity of V γ 9V δ 2 $\gamma\delta$ -T cells as measured against human epithelial-derived cancer cell lines. Here we describe the innate antitumor activity of ex vivo expanded human V γ 9V δ 2 $\gamma\delta$ -T cells against human prostate cancer cell lines and discuss this finding in the context of developing new strategies for the adoptive cellular therapy of prostate cancer.

MATERIALS AND METHODS

Tumor cell lines and antibodies.

Human prostate cell lines including DU145, LNCaP and PC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in the appropriate medium as recommended by ATCC.

Preparation of $\gamma\delta$ -T and $\alpha\beta$ -T cells from PBMC cultures

Ex-vivo expanded apoptosis resistant $\gamma\delta$ -T cells were prepared as we have previously described (26). Briefly, PBMC were isolated by Ficoll gradient centrifugation of whole

blood obtained from healthy human volunteers. Cultures were initiated at a cell density of 1×10^6 cells/mL in RPMI-1640 with 10% fetal bovine serum (HyClone, Logan, UT), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 50 μ mol/L 2-ME. On the day of culture initiation (day 0), human recombinant interferon (rIFN)- γ (1000 U/mL; Boehringer Mannheim, Indianapolis, IN); human rIL-12 (10 U/mL; R&D Systems, Minneapolis, MN), and mouse anti-human CD2 mAb clone S5.2 (1-10 μ g/mL, mouse IgG2a; Becton Dickinson, San Jose, CA) were added. Twenty-four hours later (day 1), cultures were stimulated with 10 ng/mL anti-CD3 mAb OKT3 (mouse IgG2a; Orthobiotec, Raritan, NJ) and 300 U/mL human rIL-2 (Boehringer Mannheim). Fresh medium with 10 U/mL IL-2 was added every 7 days. The expanded cultures were subjected to $\gamma\delta$ -T cell positive selection for $\gamma\delta$ -T cells, and $\gamma\delta$ -T cell depletion for $\alpha\beta$ -T cell selection by immunomagnetic column separation on day 13 or 14. Cells were first stained with an anti- $\gamma\delta$ -TCR mAb conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA) then passed through a immunomagnetic cell sorter (AutoMACS, Miltenyi Biotec) using the manufacturer's instructions. Isolated cells were washed with PBS and cultured overnight in complete RPMI medium with 100U/ml of IL-2. Purity of isolated $\gamma\delta$ -T cells and $\alpha\beta$ -T cells was assessed by flow cytometry and routinely found to be > 95 percent with > 90 percent viability.

Chromium ^{51}Cr -release cytotoxicity assay

Target cells were labeled with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ (Amersham Pharmacia Biotech, Piscataway, NJ) overnight at 37° C, after which cells were washed, trypsinized, and suspended in RPMI containing 10% FBS. Cells were then plated (2×10^3 /well) in 96-well V-bottom microtiter trays. Purified $\alpha\beta$ - or $\gamma\delta$ -T cells in varying numbers were added to target cells in a final volume of 150 μL . Trays were briefly centrifuged and then incubated for 4 hours at 37° C, after which 50 μL supernatant was removed to determine ^{51}Cr release in cpm. Percentage of specific target cell lysis was calculated as $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$. Maximum and spontaneous releases were respectively determined by adding either 0.1% Triton X-100 or culture medium alone to labeled target cells in the absence of effector cells. Data are presented as the mean (\pm SD) of triplicate samples.

Flow cytometry analysis of cells

Flow cytometry analysis was performed as we previously described (26). Briefly, cells were stained with directly fluorescence-conjugated mAbs recognizing TCR- $\delta 1$, TCR- $\delta 2$ and CD3 separately. Directly fluorescence-conjugated isotype-matched irrelevant antibodies served as controls. Analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson). Propidium iodide (PI) uptake was used to exclude non-viable cells during analysis. Data analysis was performed using CellQuest software (Becton Dickinson).

RESULTS

Ex-vivo expanded apoptosis resistant $\gamma\delta$ -T cells express primarily the V γ 9V δ 2 TCR

To determine the usage of TCR on expanded apoptosis resistant $\gamma\delta$ -T cells, total $\gamma\delta$ -T cells were first isolated from PBMC cultures by immunomagnetic column separation as described above. Purified cells were cultured for 24 hours prior to analysis to allow mAb used for sorting to dissociate. Anti-V δ 1 and V δ 2 mAb conjugated with FITC and PE respectively as well as anti-CD3 mAb with APC were incubated with the cells. After being washed, the cells were analyzed by FACS. PI negative events were gated for analysis to exclude non-viable cells. The results are shown in Figure 1 where 96% of gated cells display CD3 and V δ 2 on their surface, while essentially no V δ 1 $\gamma\delta$ -T cells were detected in the expanded cultures.

V γ 9V δ 2 $\gamma\delta$ -T cells exhibit cytotoxicity activity against some prostate tumor cells

It is established that activated V γ 9V δ 2 $\gamma\delta$ -T cells exhibit cytotoxicity activity to lymphoma cell lines. However, the ability of these cells to recognize and lyse malignant cell lines of epithelial origin has not been well described. Here, we examined cytolytic activity of apoptosis resistant V γ 9V δ 2 $\gamma\delta$ -T cells against human prostate tumor cell lines DU145, PC-3 and LNCaP. PBMC were isolated from healthy donors then cultured as described above. After 14 days, apoptosis-resistant $\gamma\delta$ -T cells were sorted to high purity utilizing the Miltenyi AutoMACS immunomagnetic cell separator. Human prostate cancer cell lines DU145, PC-3 and LNCaP were first labeled with ^{51}Cr , then incubated with $\gamma\delta$ -T cells or control $\alpha\beta$ -T cells. After a 4 hour incubation at the indicated E:T ratios, supernatants were removed to determine ^{51}Cr release in CPM. Data are expressed as the mean percent specific target lysis (\pm SD) of triplicate determinations. These studies are representative of experiments performed 3 other times. These findings indicate that apoptosis resistant $\gamma\delta$ -T cells can specifically lyse human prostate cancer cell lines DU145 and PC-3 LNCaP (Figure2).

Involvement $\gamma\delta$ TCR in the lysis of sensitive tumor cells by V γ 9V δ 2 $\gamma\delta$ -T cells

V δ 1 $\gamma\delta$ -T cells are able to kill a wide variety of epithelial derived tumor cells in vitro, as has been reported. Above, we have demonstrated that V γ 9V δ 2 $\gamma\delta$ -T cells are also able to recognize some epithelial tumor cells such as DU145 and PC-3. To determine whether the $\gamma\delta$ TCR itself is involved in the recognition and killing of tumor cells, blocking antibodies to either TCR chain V γ 9 or V δ 2 were added to effector $\gamma\delta$ -T cells prior to co-culture with tumor target cells (Figure 3). Both antibodies inhibited specific lysis, suggesting that TCR participates in the $\gamma\delta$ -T cell-mediated lysis of tumor cells. The function of TCR in antitumor was also verified in cytotoxicity assays by the addition of pan- $\gamma\delta$ TCR mAbs which recognize all $\gamma\delta$ -T cells, regardless of TCR chain expression.

Involvement of additional cell surface structures in the recognition and lysis of sensitive tumor cells by V γ 9V δ 2 $\gamma\delta$ -T cells

Similar studies were performed utilizing a number of mAbs to known surface structures likely to be involved in the interaction between cytotoxic $\gamma\delta$ -T cells and sensitive prostate cancer cell line PC-3. In a number of studies performed, only mAb to CD18 and CD54

were able to consistently inhibit $\gamma\delta$ -T cell-mediated killing of PC-3 cells, suggesting that interactions between CD18 and CD54/ICAM-1 may be involved in the recognition and killing of prostate cancer cells by V γ 9V δ 2 $\gamma\delta$ -T cells (Figure 3).

V γ 9V δ 2 $\gamma\delta$ -T cell-mediated cytotoxicity of tumor cells involves perforin/granzyme pathway

Two independent pathways, granule exocytosis and Fas-induced apoptosis have been reported in cytotoxic T lymphocytes (CTL) mediated cytotoxicity.¹³ As $\gamma\delta$ -T cells constitutively express perforin and granzymes (serine esterase) we examined whether perforin/granzyme exocytosis as a mechanism involved in lysis of tumor cells. As granule exocytosis is Ca^{2+} -dependent, studies cytotoxicity assays were performed in the presence of Ca^{2+} -chelators. As shown in Figure 4, lysis of DU145 by V γ 9V δ 2 $\gamma\delta$ -T cells was blocked by the addition of EGTA- Mg^{2+} (depletion of Ca^{2+}), but is partially restored by Ca^{2+} replacement – suggesting that V γ 9-V δ 2 $\gamma\delta$ -T cell-mediated cytotoxicity of prostate cancer cells depends involves the perforin/granzyme pathway.

Discussion

It has been well established that human V δ 1 $\gamma\delta$ -T cells can recognize and kill sensitive human tumor cells through the recognition of MHC class I-related molecules MICA and MICB which are expressed on malignant transformed cells (27). This interaction appears to occur through NKG2d, a receptor expressed by subsets of cytolytic T cells and natural killer (NK) cells. MICA and/or MICB also appear to interact directly with the V δ 1 TCR (18-20, 28).

In this current study, we report that ex vivo expanded V γ 9V δ 2 $\gamma\delta$ -T cells recognize and kill epithelial-derived tumor cells in a $\gamma\delta$ TCR-dependent manner. However, it is still unclear whether MICA and/or MICB are directly involved in this process. Indeed, we observed no difference (data not shown) in MICA and MICB expression on cells which were sensitive to V γ 9V δ 2 $\gamma\delta$ -T cell-mediated killing (DU145 and PC-3) and cells that were resistant to killing (LnCap). We did observe however that expression of CD54/ICAM-1 was impaired in the cell line LNCaP which was not killed by V γ 9V δ 2 $\gamma\delta$ -T cells (data not shown), this suggesting that ICAM-1 interactions with ligands expressed on V γ 9V δ 2 $\gamma\delta$ -T cells may be important in the process of recognition and killing of sensitive tumor target cells. This is supported by our findings that blocking antibodies to either CD18 or CD54/ICAM-1 inhibited $\gamma\delta$ -T cell-mediated tumor cell lysis. Studies to more directly address this issue are currently underway and will be reported separately.

Though by no means exhaustive, our finding that ex vivo expanded, apoptosis-resistant human $\gamma\delta$ -T cells can recognize and kill human prostate cancer cells is important for a number of reasons. First, this finding is consistent with the emerging model that human $\gamma\delta$ -T cells can indeed recognize and lyse a wide variety of human epithelial-derived tumors. Importantly, here we show that V γ 9V δ 2 $\gamma\delta$ -T cells are quite capable of killing epithelial-derived tumor cells, a function that is often more associated with V δ 1 $\gamma\delta$ -T cells. Second, these findings in addition to our previous studies establish that we can

indeed expand large numbers of prostate cancer-reactive V γ 9V δ 2 $\gamma\delta$ -T cells, a finding that has never been reported. These two points taken together provide a rationale – which is both biologically sound and practical – for proposing further studies to determine how human $\gamma\delta$ -T cells might be exploited for the adoptive immunotherapy of prostate cancer.

These types of studies eventually may allow us to address several basic questions which cannot currently be addressed. For example, presuming we can routinely accomplish the clinical-scale expansion of tumor-reactive $\gamma\delta$ -T cells from patients with prostate cancer. Will infusing supra-physiological numbers of these $\gamma\delta$ -T cells restore or augment innate immune responses against tumors, thus moderating tumor growth or progression? Can these $\gamma\delta$ -T cells be utilized alone or will they best be utilized in conjunction with standard hormone, chemotherapy or radiation-based treatments? Only properly designed future clinical trials – based largely upon these such findings reported here – will be able to adequately address these issues.

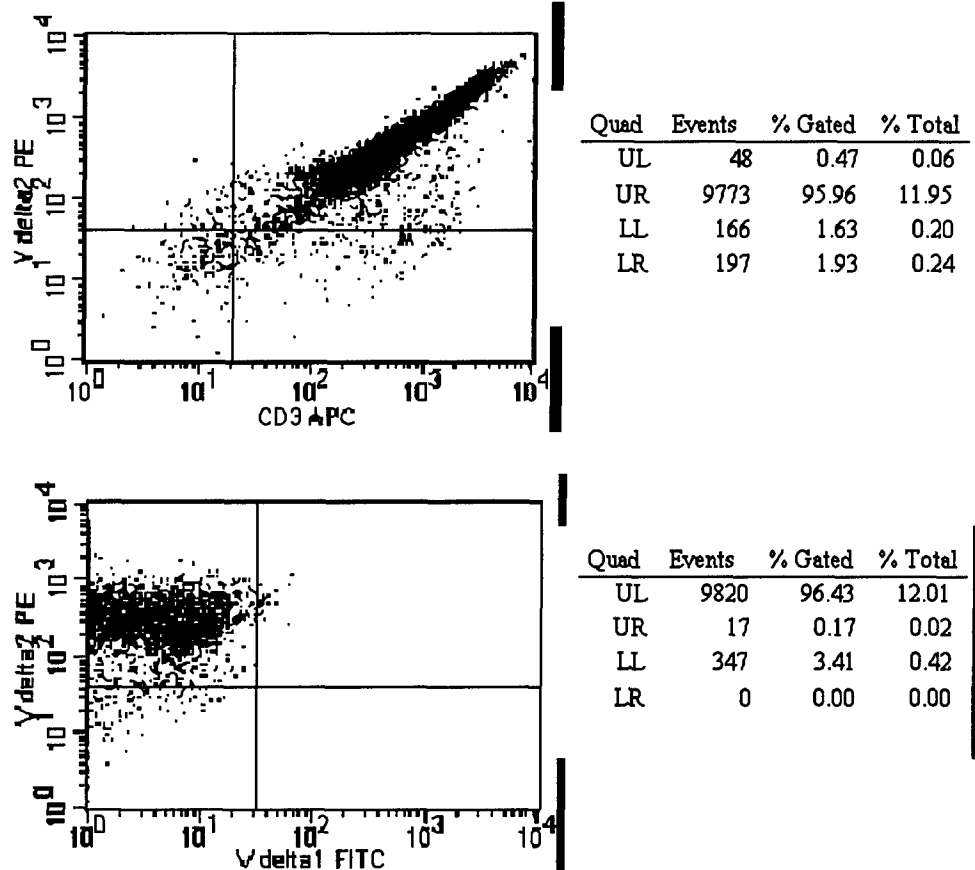


Figure 1. Composition of ex-vivo expanded apoptosis resistant $\gamma\delta$ -T cells with respect to express TCR usage. Total $\gamma\delta$ -T cells were first isolated from PBMC cultures by immunomagnetic column separation. Purified cells were cultured for 24 hours prior to analysis to allow mAb used for sorting to dissociate. Anti-V δ 1 and V δ 2 mAb conjugated with FITC and PE respectively as well as anti-CD3 mAb with APC were incubated with the cells. After being washed, the cells were analyzed by FACS. PI negative events were gated for analysis to exclude non-viable cells. Percentage of cells in the corresponding quadrant locations are shown. Result are representative of experiments performed at least three separate times using PBMC derived from healthy donors.

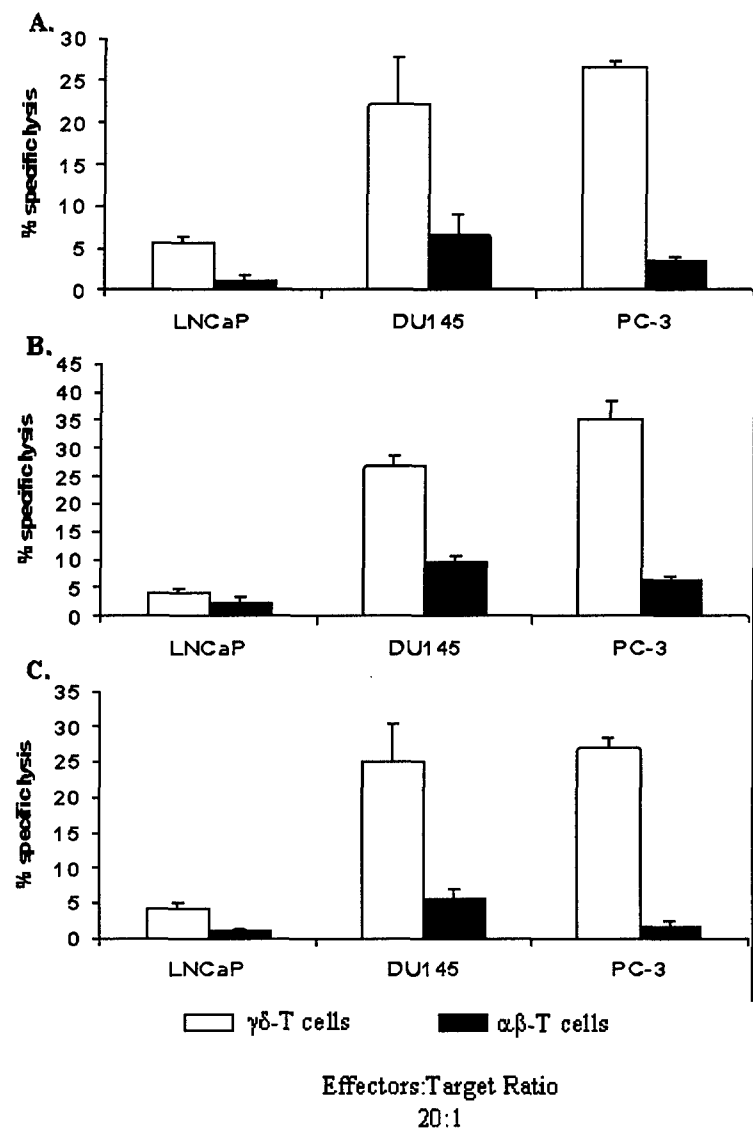


Figure 2. Antitumor activity of V γ 9V δ 2 $\gamma\delta$ -T cells against human prostate cancer cell lines. PBMC were isolated from healthy donors then cultured as described above. After 14 days, apoptosis-resistant $\gamma\delta$ -T cells were sorted to high purity utilizing the Miltenyi AutoMACS immunomagnetic cell separator. Human prostate cancer cell lines DU145, PC-3 and LNCaP were first labeled with ^{51}Cr , then incubated with $\gamma\delta$ -T cells or control $\alpha\beta$ -T cells. After a 4 hour incubation at the indicated E:T ratios, supernatants were removed to determine ^{51}Cr release in CPM. Data are expressed as the mean percent specific target lysis (\pm SD) of triplicate determinations. These studies are representative of experiments performed at least 3 other times.

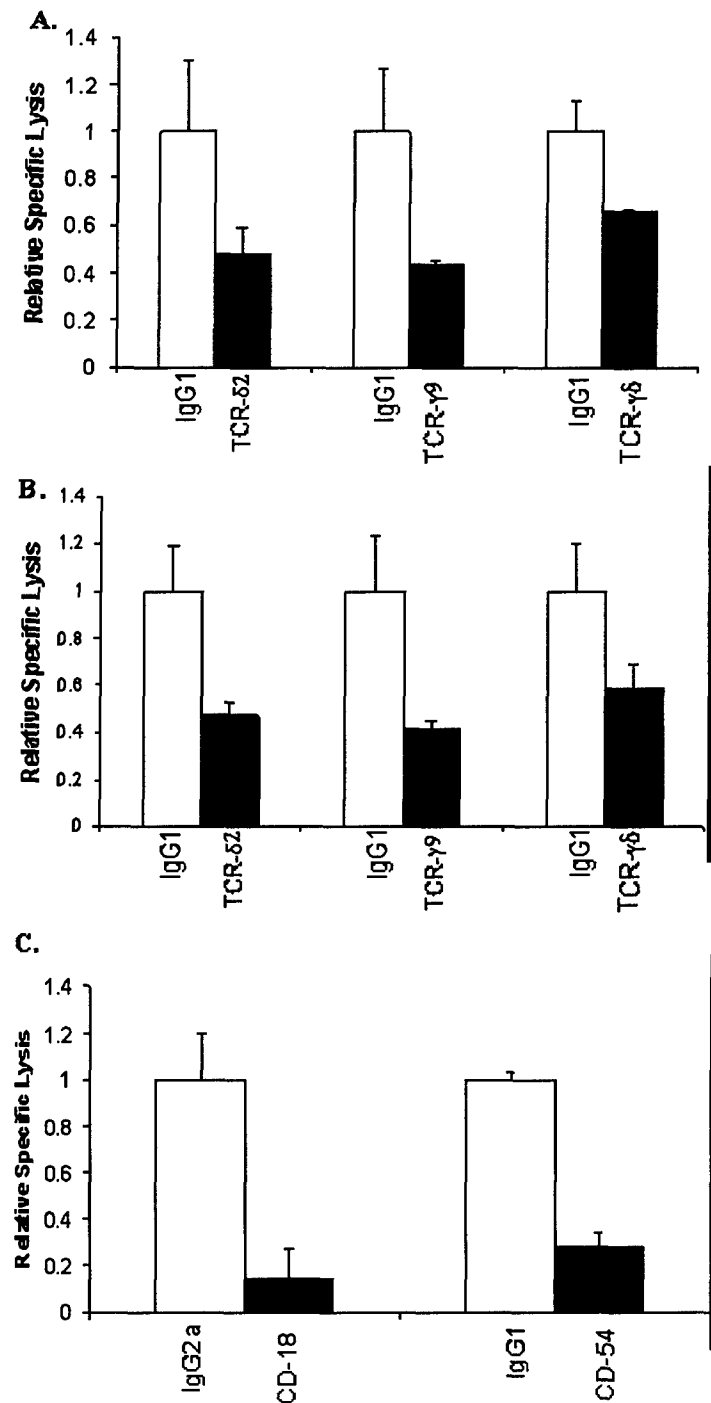


Figure 3. Antibodies to CD18 and CD54/ICAM inhibit V $\gamma 9$ V $\delta 2$ $\gamma \delta$ -T cell-mediated tumor lysis. Cytotoxicity assays were performed in the presence of anti-TCR-V $\delta 2$, anti-TCR-V $\gamma 9$ or anti-TCR- $\gamma \delta$ mAbs (or corresponding isotype controls). Effector $\gamma \delta$ -T cells were isolated as described and co-cultured with ^{51}Cr -labeled cell lines A) DU-145 or B) PC-3 at an E:T ratio or 10:1. Similarly in C), effector $\gamma \delta$ -T cells were co-cultured with ^{51}Cr -labeled PC-3 cells with the addition of mAb against CD54/ICAM-1 or CD18 (or isotype control). Relative tumor specific lysis (\pm SD of triplicate determinations) in the presence of each specific antibody was calculated by comparing with its corresponding isotype control. Studies are representative of experiments performed at least 3 other times.

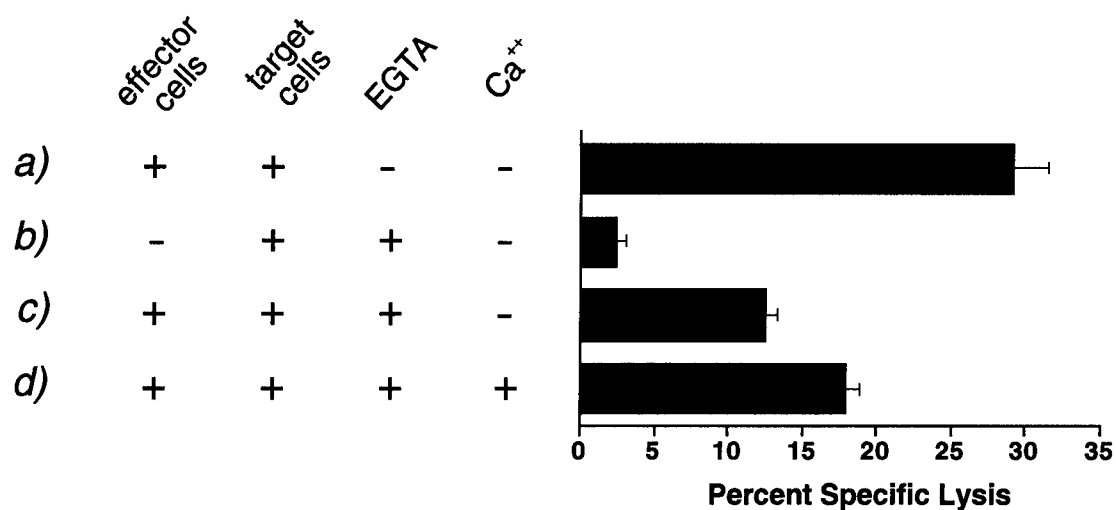


Figure 4. Cytolysis of prostate cancer cells by V γ 9V δ 2 $\gamma\delta$ -T cells involves the perforin/granzyme pathway. Standard 4 hour cytotoxicity assays were performed using ex vivo expanded V γ 9V δ 2 $\gamma\delta$ -T cells as effector cells and ^{51}Cr -labeled DU145 cells as target cells (E:T ratio, 10:1). a) Effector $\gamma\delta$ -T cells co-cultured with ^{51}Cr -labeled cell DU145 in RPMI; b) ^{51}Cr -labeled cell DU145 cultured without $\gamma\delta$ -T cells; c) Effector $\gamma\delta$ -T cells co-cultured with ^{51}Cr -labeled cell DU145 in the absence of Ca^{2+} (depleted by the addition of EGTA); d) Effector $\gamma\delta$ -T cells co-cultured with ^{51}Cr -labeled cell DU145 with Ca^{2+} replaced.

REFERENCES

1. Fong, L., Brockstedt, D., Benike, C., Breen, J. K., Strang, G., Ruegg, C. L., and Engleman, E. G. Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy, *Journal of Immunology*. 167: 7150-6, 2001.
2. Inoue, Y., Takaue, Y., Takei, M., Kato, K., Kanai, S., Harada, Y., Tobisu, K., Noguchi, M., Kakizoe, T., Itoh, K., and Wakasugi, H. Induction of tumor specific cytotoxic T lymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide, *Journal of Urology*. 166: 1508-13, 2001.
3. Mercader, M., Bodner, B. K., Moser, M. T., Kwon, P. S., Park, E. S., Manecke, R. G., Ellis, T. M., Wojcik, E. M., Yang, D., Flanigan, R. C., Waters, W. B., Kast, W. M., and Kwon, E. D. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer, *Proceedings of the National Academy of Sciences of the United States of America*. 98: 14565-70, 2001.
4. Mincheff, M., Altankova, I., Zoubak, S., Tchakarov, S., Botev, C., Petrov, S., Krusteva, E., Kurteva, G., Kurtev, P., Dimitrov, V., Ilieva, M., Georgiev, G., Lissitchkov, T., Chernozemski, I., and Meryman, H. T. In vivo transfection and/or cross-priming of dendritic cells following DNA and adenoviral immunizations for immunotherapy of cancer--changes in peripheral mononuclear subsets and intracellular IL-4 and IFN-gamma lymphokine profile, *Critical Reviews in Oncology-Hematology*. 39: 125-32, 2001.
5. Rini, B. I. and Small, E. J. Immunotherapy for prostate cancer, *Current Oncology Reports*. 3: 418-23, 2001.
6. Small, E. J., Fratesi, P., Reese, D. M., Strang, G., Laus, R., Peshwa, M. V., and Valone, F. H. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells, *Journal of Clinical Oncology*. 18: 3894-903, 2000.
7. Tjoa, B. A. and Murphy, G. P. Progress in active specific immunotherapy of prostate cancer, *Seminars in Surgical Oncology*. 18: 80-7, 2000.
8. Tjoa, B. A., Simmons, S. J., Elgamal, A., Rogers, M., Ragde, H., Kenny, G. M., Troychak, M. J., Boynton, A. L., and Murphy, G. P. Follow-up evaluation of a phase II prostate cancer vaccine trial, *Prostate*. 40: 125-9, 1999.
9. Peshwa, M. V., Shi, J. D., Ruegg, C., Laus, R., and van Schooten, W. C. Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide, *Prostate*. 36: 129-38, 1998.
10. Sanda, M. G., Restifo, N. P., Walsh, J. C., Kawakami, Y., Nelson, W. G., Pardoll, D. M., and Simons, J. W. Molecular characterization of defective antigen processing in human prostate cancer, *J Natl Cancer Inst*. 87: 280-5., 1995.
11. Elzey, B. D., Siemens, D. R., Ratliff, T. L., and Lubaroff, D. M. Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors, *International Journal of Cancer*. 94: 842-9, 2001.
12. Lodge, P. A., Childs, R. A., Monahan, S. J., McLean, J. G., Sehgal, A., Boynton, A. L., Salgaller, M. L., and Murphy, G. P. Expression and purification of prostate-specific membrane antigen in the baculovirus expression system and recognition by

prostate-specific membrane antigen-specific T cells, *Journal of Immunotherapy*. 22: 346-55, 1999.

13. Meidenbauer, N., Gooding, W., Spitler, L., Harris, D., and Whiteside, T. L. Recovery of zeta-chain expression and changes in spontaneous IL-10 production after PSA-based vaccines in patients with prostate cancer, *British Journal of Cancer*. 86: 168-78, 2002.

14. Saffran, D. C., Reiter, R. E., Jakobovits, A., and Witte, O. N. Target antigens for prostate cancer immunotherapy, *Cancer & Metastasis Reviews*. 18: 437-49, 1999.

15. Willis, R. A., Bowers, W. J., Turner, M. J., Fisher, T. L., Abdul-Alim, C. S., Howard, D. F., Federoff, H. J., Lord, E. M., and Frelinger, J. G. Dendritic cells transduced with HSV-1 amplicons expressing prostate-specific antigen generate antitumor immunity in mice, *Human Gene Therapy*. 12: 1867-79, 2001.

16. Blades, R. A., Keating, P. J., McWilliam, L. J., George, N. J., and Stern, P. L. Loss of HLA class I expression in prostate cancer: implications for immunotherapy, *Urology*. 46: 681-6; discussion 686-7, 1995.

17. Bander, N. H., Yao, D., Liu, H., Chen, Y. T., Steiner, M., Zuccaro, W., and Moy, P. MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma, *Prostate*. 33: 233-9, 1997.

18. Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K. H., and Spies, T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB, *Proceedings of the National Academy of Sciences of the United States of America*. 96: 6879-84, 1999.

19. Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L., and Spies, T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA [see comments], *Science*. 285: 727-9, 1999.

20. Groh, V., Steinle, A., Bauer, S., and Spies, T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells, *Science*. 279: 1737-40, 1998.

21. Triebel, F. and Hercend, T. Subpopulations of human peripheral T gamma delta lymphocytes.[see comment], *Immunology Today*. 10: 186-8, 1989.

22. Kunzmann, V., Bauer, E., Feurle, J., Weissinger, F., Tony, H. P., and Wilhelm, M. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma.[see comment], *Blood*. 96: 384-92, 2000.

23. Wilhelm, M., Kunzmann, V., Eckstein, S., Reimer, P., Weissinger, F., Ruediger, T., and Tony, H. P. Gammadelta T cells for immune therapy of patients with lymphoid malignancies, *Blood*. 102: 200-6, 2003.

24. Ottones, F., Liautard, J., Gross, A., Rabenoelina, F., Liautard, J. P., and Favero, J. Activation of human Vgamma9Vdelta2 T cells by a Brucella suis non-peptidic fraction impairs bacterial intracellular multiplication in monocytic infected cells, *Immunology*. 100: 252-8, 2000.

25. Hayday, A. C. [gamma][delta] cells: a right time and a right place for a conserved third way of protection, *Annual Review of Immunology*. 18: 975-1026, 2000.

26. Lopez, R. D., Xu, S., Guo, B., Negrin, R. S., and Waller, E. K. CD2-mediated IL-12-dependent signals render human gamma-delta T cells resistant to mitogen-induced apoptosis, permitting the large-scale ex vivo expansion of functionally distinct lymphocytes: implications for the development of adoptive immunotherapy strategies, *Blood*. 96: 3827-3837, 2000.

27. Wu, J., Groh, V., and Spies, T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells, *Journal of Immunology*. 169: 1236-40, 2002.
28. Crowley, M. P., Fahrner, A. M., Baumgarth, N., Hampl, J., Gutgemann, I., Teyton, L., and Chien, Y. A population of murine gammadelta T cells that recognize an inducible MHC class Ib molecule, *Science*. 287: 314-6, 2000.